Heat Stability of the Bile Salt-Stimulated Lipase in Human Milk Fortified with Sodium Taurocholate

H. L. PAN, C. W. DILL, E. S. ALFORD, S. L. DILL, C. A. BAILEY, R. L. RICHTER and C. GARZA

Department of Animal Science, Texas A&M University, College Station, Texas 77843 and Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

(Received for publication February 17, 1987)

ABSTRACT

Time-temperature relationships for heat-inactivation of the bile salt-stimulated lipase activity were compared in whole human milk and in the same product fortified to 9 mM/ml with sodium taurocholate. Heat treatments were varied from 45 to 70°C for times ranging from 15 s to 40 min. Enzyme activity was more heat stable in human milk fortified with taurocholate than in control samples. The temperature required for the onset of heat inactivation at 30-min holding time was increased from 45°C for control samples to 60°C following addition of taurocholate. A temperature differential of approximately 12°C was required in the fortified milks to produce inactivation equivalent to that observed in the control milks over the heating range studied.

Bile salt-stimulated (BSS) lipase activity in human milk is important to lipid digestion by the neonate (7,16,18,22,23). Indeed, Olivecrona and Hernell (12) concluded that the BSS lipase in human milk is essential for proper fat absorption by neonates, and particularly low birth weight infants. Thus the stability of this enzyme may be a critical factor in development of a protocol for heat processing and banking human milk for this special group as a practical alternative to the previously suggested (18) fortification of infant diets with this lipase component.

The mode of action of bile salts in stimulating BSS lipase activity is not clear. Brockerhoff and Jensen (4) reviewed effect of bile salts on lipolysis and concluded that binding of bile salts to the lipase was essential to subsequent orientation of the enzyme at the oil-water interface. Wang (20) observed that the purified enzyme was selective in its bile salt activation requirement. The 7-hydroxy group of primary bile salts appeared essential. He also discounted the argument that activation was due to a substrate solubilizing effect. Hernell and Olivecrona (10) observed that primary bile salts produced a marked activation of the lipase enzyme, yet both primary and secondary bile salts should produce a significant detergent action.

Solubilized bile salts do behave as surfactants in biological systems. Brockerhoff (3) determined that surfactants protect the lipase enzyme's native structure by preventing a loss of tertiary structure. He further demonstrated that taurocholate prevented denaturation of pancreatic lipase at the oil-water interface.

Pan et al. (14) performed a comprehensive time-temperature study of heat-inactivation of the BSS-stimulated lipase activity in human milk. They concluded that this enzyme was more heat sensitive than the lipase in bovine milk, and so it would follow that heat treatments sufficient to pasteurize human milk should completely denature this enzyme (11). Hernell (9) demonstrated that the BSS lipase in human milk was less sensitive to heat in the presence of taurocholate. The purpose of this investigation was to provide more comprehensive information on the sparing effect of taurocholate on the bile salt-stimulated lipase activity in human milk over a range of heating times and temperatures.

MATERIALS AND METHODS

Sample collection
Ten pools of human milk were collected by trained personnel with the Human Milk Bank and Distribution Center, Baylor College of Medicine, Houston, TX. Milk samples were collected, between 10:00 a.m. and 12:00 noon, with an Egnell (Cary, IL) breast pump and represent the complete secretion of one breast. Samples were immersed immediately in an ice-water mixture. The cooled samples were transported to Texas A&M University, at which time the samples were pooled. Each pool contained at least 600 ml of whole milk and represented milk from at least 6 donors.

Heating protocol
Milk samples were heated to temperatures between 45 and 60°C with times varying from 15 s to 40 min in the heating system.
Human milk lipase described previously (13,14). The heating system consisted of two thin stainless steel plates spaced to contain a 40-ml volume of milk. Portions of pooled milk were pre-warmed to 30°C to minimize the time required for samples to reach the final indexed temperature in the thin-plate heating apparatus. Three-milliliter portions were removed sequentially from the heating vessel at the end of each holding period with a glass syringe, and immersed in an ice-water mixture.

A preliminary study was made to determine the optimum levels of sodium taurocholate required to stabilize the lipolytic activity. Samples of pooled milk were heated at 60°C for 2 min because previous observations revealed that this heat treatment was just sufficient to completely inactivate the bile salt-stimulated enzyme activity (14). Zero to 37.2 mM of sodium taurocholate were added to a liter of milk before heat treatment.

**Measurement of lipase activity**

Bile salt-stimulated lipase activity was measured by a modification of the method of Parry et al. (15), using a pH-stat titrator (Metrohm-Herisau). The enzyme substrate was 10% triolein (Sigma) in a 10% (w/w) gum arabic (Sigma) solution. Five milliliters of substrate, 0.2 ml of 2.85 M NaCl and 0.1 ml of 20% (w/v) aqueous sodium taurocholate (Sigma) were placed in the titration vessel. The mixture was maintained at 37°C. One milliliter of milk was added and the dispersion was adjusted to pH 8.8. The volume of 0.01 N NaOH required to maintain pH 8.8 was recorded as a function of time. A unit of activity was defined as the μmoles of free fatty acid min⁻¹ ml⁻¹. All data were analyzed by analysis of variance, and a general linear model (8) was used to calculate lipolytic regression functions.

**RESULTS**

Results of the preliminary experiment establishing the optimal sparing effect of taurocholate are summarized in Fig. 1. The absence of lipase activity at the zero level of taurocholate was expected because of the heat treatment chosen (60°C for 2 min). The residual enzyme activity increased rapidly as taurocholate concentration was increased from 0 to 3 mM, and then at a slower rate until a maximum protective effect was observed at approximately 9 mM. At taurocholate concentrations above 9 mM the stabilized lipase activity decreased about 20% from the maximum to a plateau value of about 11 enzyme units.

For the comprehensive study of the stabilizing effect of taurocholate, a bile salt concentration of 10 mM was added to test milks before heat treatment. Heating temperatures of 60, 63, 65, 67 and 70°C were used, and heating times ranged from 15 s to 40 min. The results for milks containing no added taurocholate are in Fig. 2, while the results for milk samples containing sodium taurocholate are in Fig. 3. The results for the control samples (Fig. 2) are very similar to those reported previously (14). No change was noted at 45°C during a 30-min incubation and a very rapid loss of activity occurred at 60°C. In contrast, a much greater stability of lipase activity was noted in samples containing added taurocholate. An initial decrease of about 10% activity was noted during the first 15 s at 60°C, followed by a slower decrease.
Figure 3. Residual bile salt-stimulated lipase activity in human milks fortified with sodium taurocholate before heating at 60 (○○), 63 (■■), 65 (■■), 67 (○○), and 70°C (∆∆). Heating Time (min)

Figure 4. Time-temperature relationships for inactivation of the bile salt-stimulated lipase activity in human milk (A), human milk fortified with sodium taurocholate prior to heat treatment (B), and the lipolytic activity in bovine milk (C).

... during further incubation at this temperature to a time of 40 min. Total inactivation of the lipolytic activity was observed after 40 min at 65°C and 10 min at 67°C.

Significantly greater levels of heat were required to inactivate the BSS lipase enzyme in human milks with added sodium taurocholate than in milks with no added taurocholate. This effect is most easily visualized in Fig. 4, which represents minimum time-temperature combinations required for complete inactivation of the BSS lipase activity in human milks with and without sodium taurocholate. The time and temperature relationship for total inactivation of bovine milk lipase is included as a means of validating our heating system, since the time and temperature relationship for total inactivation of bovine milk lipase closely duplicates the data of Hetrick and Tracy (11) obtained with commercially available heating equipment designed for processing large quantities of bovine milk. The time-temperature curves for human milks with and without taurocholate (Fig. 4) are parallel; however, a temperature differential of about +12°C is required to produce a level of inactivation in milk with added taurocholate equivalent to that observed in control milks at a given level of heat treatment. Arrhenius activation energies (17) for irreversible inactivation of the BSS lipase in fortified and control milks were calculated to be 1.57 x 10^5 cal/mole for the control milks and 2.35 x 10^5 cal/mole in milks fortified with sodium taurocholate.

DISCUSSION

Hernell (9) observed that heat inactivation at 50°C of the BSS lipase in an acetone powder of human milk was impeded when the powder was dispersed in buffer containing 5 mM sodium taurocholate per liter, as compared to the buffered samples free of taurocholate. Results obtained in the present study confirm the protective effect of taurocholate using fresh human milk heated over a range of times and temperatures. When the results for samples heated at 50°C were compared to those of Hernell (9), this common heat treatment was less destructive toward the BSS lipase in fresh milk than in an acetone powder of milk dispersed in veronal buffer. This conclusion disregards any differences in heating systems. These data did not reflect a loss of BSS lipolytic activity in fresh milks at temperatures below 60°C when the milks had been fortified with sodium taurocholate.

Results reported here are not definitive to the extent that a full explanation of the action of bile salts in protecting the lipase against heat denaturation, or in stimulating its activity, is possible. However, certain points are clear. First, the action of sodium taurocholate not only stimulates the lipolytic action of the major lipase in human milk (9), but also is very significant in terms of protection of the molecule from heat denaturation. Second, the Hetrick and Tracy (11) plots of the data show that heat denaturation of the lipase molecule, with and without taurocholate, follow two distinctly different processes with considerably more heat required to denature the enzyme in milks fortified with taurocholate.

One explanation of these results, which would be favored by the data reported by Wang (20,21), would be that a conjugate formed between the lipase and bile salts causes the lipase molecule to resist unfolding and denaturation due to heat, or that the unfolding of lipase was more reversible in the...
presence of taurocholate. The detergent would likely prevent intermolecular interactions upon cooling of the unfolded enzyme, and thus favor intramolecular interactions, or partial renaturation, of the enzyme. A third plausible explanation is that the lipase exists in human milk in a bound, or compartmentalized, state that impairs its ability to hydrolyze triacylglycerols. The subsequent action of bile salts, acting as detergents, releases the enzyme from this compartment. It is generally known that small enzyme molecules are more heat stable than are oligomeric forms or molecules that are bound to other proteins or even to their substrates (17). This compartmentation of the native enzyme also would explain differences in molecular weights reported in the literature (1,21). When fresh human milk is heated, action of heat may result either in a denaturation of the enzyme within the compartment, or an induration of the compartment about the enzyme making it unavailable for its normal physiological role. Thus, when the milk is heated in the presence of taurocholate, a normal heat denaturation curve for the lipase is observed for the free enzyme after its release from its native compartment by the dispersing action of bile salts (2,5,19).

Further work is needed to define the locus of the BSS lipase in its native state in human milk and to better correlate that information with changes in chemical and physiological parameters brought about by the presence of bile salts in the lipolytic system. These results suggest the distinct possibility of heat pasteurization of human milk in a manner to preserve a major portion of the BSS lipase activity which appears so vital to the low birth weight neonate.

REFERENCES